

Role of oxidative stress-induced systemic and cavernosal molecular alterations in the progression of diabetic erectile dysfunction

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Abstract

Background: Erectile dysfunction (ED) is a prevalent complication of diabetes, and oxidative stress is an important feature of diabetic ED. Oxidative stress-induced damage plays a pivotal role in the development of tissue alterations. However, the deleterious effects of oxidative stress in the corpus cavernosum with the progression of diabetes remain unclear. The aim of this study was to evaluate systemic and penile oxidative stress status in the early and late stages of diabetes.

Methods: Male Wistar streptozotocin-diabetic rats (and age-matched controls) were examined 2 (early) and 8 weeks (late) after the induction of diabetes. Systemic oxidative stress was evaluated by urinary H_2O_2 and the ratio of circulating reduced/oxidized glutathione (GSH/GSSG). Penile oxidative status was assessed by H_2O_2 production and 3-nitrotyrosine (3-NT) formation. Cavernosal endothelial nitric oxide synthase (eNOS) was analyzed by quantitative immunohistochemistry. Dual immunofluorescence was also performed for 3-NT and α -smooth muscle actin (α -SMA) and eNOS- α -SMA. **Results:** There was a significant increase in urinary H_2O_2 levels in both diabetic groups. The plasma GSH/GSSG ratio was significantly augmented in late diabetes. In cavernosal tissue, H_2O_2 production was significantly increased in late diabetes. Reactivity for 3-NT was located predominantly in cavernosal smooth muscle (SM) and was significantly reduced in late diabetes. Quantitative immunohistochemistry revealed a significant decrease in eNOS levels in cavernosal SM and endothelium in late diabetes.

Conclusions: The findings indicate that the noxious effects of oxidative stress are more prominent in late diabetes. Increased penile protein oxidative modifications and decreased eNOS expression may be responsible for structural and/or functional deregulation, contributing to the progression of diabetes-associated ED.

Keywords: 3-nitrotyrosine, diabetes, endothelial nitric oxide synthase, erectile dysfunction, oxidative stress

Introduction

Erectile dysfunction (ED) is a common clinical problem in diabetes that has detrimental impacts on the quality of life of men and their partners.¹ The incidence of ED in men with diabetes is approximately threefold higher than in the general population.^{2,3} In fact, men with diabetes have a greater prevalence and earlier onset of ED than the general population, with ED affecting up to 75% of

all diabetic patients.^{2,3} The pathophysiology of diabetic ED is multifactorial, consisting predominantly of vascular and neurological insults as a result of hyperglycemia induced-metabolic derangements.⁴ Chronic elevated glucose concentrations have been reported to promote the generation of reactive oxygen (ROS) and nitrogen species (RNS), and to induce the formation of advanced glycation end-products (AGE).⁵ In turn, this may cause changes in the bioavailability of neuronal- and endothelial-derived nitric oxide (NO), which may impair vasorelaxation mechanisms in the diabetic corpus cavernosum (CC).^{6,7} Reductions in NO may be responsible for the lower response of diabetic men with ED to treatment with oral phosphodiesterase-5 inhibitors.⁸ In addition to changes in penile vasorelaxation, accumulation of ROS is associated with decreased antioxidant levels, lipid peroxidation, and DNA damage, which may directly damage cavernosal cellular structure and function.^{9,10} The deleterious effects of ROS in ED are supported by studies in experimental models showing that treatment with antioxidants reduces superoxide production (O_2^-) and increases endothelial NO synthase (eNOS), ameliorating diabetic ED.^{9–12}

Even though the link between oxidative stress and diabetic ED seems irrefutable, the redox mechanisms in cavernosal tissue, which may contribute to the development and progression of diabetic ED, remain unknown. Basic research studies of the effects of ROS in diabetic penile tissue have focused primarily on O_2 production and its relevance in diabetes-related ED.⁷ However, another important ROS molecule, namely H_2O_2 , has not been assessed in diabetic CC; H_2O_2 is a powerful oxidizing agent, produced after spontaneous or superoxide dismutase (SOD)-catalysed dismutation of O_2^- , as well as by other enzymatic reactions.¹³ Unlike O_2^- , which remains at the site of production, H_2O_2 can diffuse across membranes and through the cytosol, potentially causing oxidative damage to most cell compartments, including DNA, membrane lipids, and proteins. Oxidation modifications of proteins, including protein nitration, may contribute to altered cell and tissue homeostasis. Protein nitration is a chemical process in which nitro groups ($-NO_2$) are preferentially introduced in certain amino acids, including tyrosines. In fact, protein nitration of

tyrosine residues to 3-nitrotyrosine (3-NT) represents an oxidative post-translational modification of key properties of the amino acid, including redox potential, hydrophobicity, and volume, therefore altering protein structure and function.¹⁴

The formation of 3-NT is mediated by RNS, such as peroxynitrite anion ($ONOO^-$) and nitrogen dioxide (NO_2), formed as secondary products of NO metabolism in the presence of oxidants including O_2^- , H_2O_2 , and transition metal centers.¹⁵ These oxidative reactions involving NO, further decrease its bioavailability, aggravating endothelial dysfunction and severely affecting vasorelaxation events. Increased systemic and tissue levels of 3-NT have been reported in diabetes.^{16,17} However, the presence of 3-NT in diabetic erectile tissue has not been thoroughly evaluated with the progression of the disease. In addition to direct inactivation of NO when promoting 3-NT formation, cavernosal reductions in NO may be related to changes in eNOS expression,¹⁸ contributing to a more severe impairment of diabetic CC endothelial-dependent and smooth muscle (SM) relaxation.

Taking these issues into consideration, our intention was to investigate oxidative stress-induced modifications in diabetic CC with the progression of the disease that may contribute to the degeneration of cellular and molecular mechanisms, essential for tissue homeostasis and erectile function. Thus, the aim of the present study was to characterize the systemic and penile redox status in early (2 weeks) and late (8 weeks) stages of streptozotocin (STZ)-induced type 1 diabetes. In addition, we evaluated the impact of the duration of diabetes on cavernosal production of H_2O_2 and 3-NT formation and whether the progression of the disease significantly changes eNOS expression. Elucidating oxidative stress-induced pathways involved in the progression of diabetic ED will provide a better insight into the deleterious effects of diabetes both systemically and at the penile level.

Methods

Animals

All experimental and animal handling procedures were conducted in accordance with the ethical guidelines proposed by the Portuguese General Veterinary Directorate (DGV) in the Directive of 24 November 1986 (86/609/EEC), with the recommendations of 18 June 2007 (2007/526/EC) proposed by the Council of the European Communities. In all, 40 male Wistar rats (280–350 g; Charles River Laboratories, Barcelona, Spain) were maintained in a 12-h day–light cycle with free access to

food and tap water. Rats were initially randomly divided into two groups ($n = 20$ in each), a diabetic group and an age-matched healthy control group. Type 1 diabetes was induced by a single intraperitoneal injection of 50 mg/kg streptozotocin (STZ; Sigma-Aldrich, Sintra, Portugal) dissolved in 0.1 mol/L citrate buffer (pH 4.5); age-matched control rats were injected with vehicle. Diabetes (glucose levels >250 mg/dL) was successfully induced and confirmed by Accu-Check glucose measurements (Roche Diagnostics, Mannheim, Germany). The effects of oxidative stress were evaluated in early (2 weeks) and late (8 weeks) diabetes, as well as in age-matched controls ($n = 10$ in each group). At both 2 and 8 weeks after STZ or vehicle injection, rats were placed in metabolic cages and urine was collected over a 24-h period. Rats were then killed, blood samples collected, and their penises excised. A fragment of penile tissue was weighed (80–120 mg) and used to assess H_2O_2 release; the remaining tissues were stored at -80°C for subsequent protein extraction or embedding in OCT.

Quantification of H_2O_2

In the present study, H_2O_2 levels were evaluated in diluted urine samples using the Amplex Red Hydrogen Peroxide Assay kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, urine was diluted in kit reaction buffer (1 : 10 control animals and 1 : 50 diabetic animals) according to the instructions provided with the kit. Fluorescence intensity was then measured in a multiplate reader (Spectramax Gemini XS; Molecular Devices, Sunnyvale, CA, USA). Urinary H_2O_2 production is given as nmol H_2O_2 /mg creatinine. Using the same method, H_2O_2 release was evaluated in penile tissue fragments that had been incubated for 90 min at 37°C in oxygenated Krebs²–HEPES buffer (composition [in mmol/L]: NaCl 118.0; KCl 4.5; $CaCl_2$ 2.5; $MgCl_2$ 1.20; K_2HPO_4 1.2; $NaHCO_3$ 25.0; Na-HEPES 25.0; glucose 5.0, pH 7.4). Levels of H_2O_2 in CC samples are expressed as nmol H_2O_2 /mg tissue.

Determination of the reduced/oxidized glutathione ratio

The reduced/oxidized glutathione (GSH/GSSG) ratio was evaluated in blood samples chromatographically using a commercially available kit (Immundiagnostik, Bensheim, Germany) and a Shimadzu LC system (Shimadzu, Kyoto, Japan) equipped with an LC 20AD pump, a DGU-20A5 degasser, and an RF-10AXL fluorescence detector (FLD). Separation was performed on a Luna column (C18, 5- μm particle size, 4.60×150 mm; Phenomenex, Torrance, CA, USA) at room temperature ($20 \pm 1^\circ\text{C}$). The injected volume was 20 μL and LC solution software version 2.1 (Shimadzu) was used for

control and data processing. The chromatographic conditions used were as described in the manual that came with the kit. Fluorescence intensity was measured at excitation and emission wavelengths of 385 and 515 nm, respectively, the flow rate was 1.0 mL/min, and chromatograms were recorded.

Western blotting for 3-NT

Penile protein extraction and quantification was performed as described previously.¹⁹ Briefly, 8 μg total protein was separated by electrophoresis on a 10% sodium dodecyl sulfate–polyacrylamide gel. After transfer, nitrocellulose membranes were incubated overnight with the cross-reactive rabbit anti-3-NT polyclonal antibody (1 : 1000 dilution; AB5411; Upstate, Millipore, Temecula, CA, USA). Antibody binding was detected after incubation with the respective secondary horseradish peroxidase-conjugated antibody (1 : 2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and using enhanced chemiluminescence (ECL kit; Amersham Biosciences, Buckinghamshire, UK). Membranes were stripped and re-probed with a rabbit anti-human β -actin antibody (1 : 3000; Abcam, Cambridge, UK), as described for 3-NT. Changes in 3-NT protein expression were quantified by densitometry (Vision Works LS Software; UVP, Upland, CA, USA). Results are expressed as mean relative intensity and were normalized against the β -actin protein signal.

Dual immunofluorescence

Expression of 3-NT and eNOS was assessed by dual immunofluorescence for both proteins and α -smooth muscle actin (α -SMA).²⁰ Briefly, sections were incubated with a mixture of the cross-reactive primary antibodies, namely: rabbit anti-3-NT (1 : 400; Upstate, Millipore) and mouse anti-human α -SMA (1 : 500; clone ASM; Chemicon International, Temecula, UK); and rabbit anti-human eNOS (1 : 85; Abcam) and mouse anti-human α -SMA (1 : 500; clone ASM; Chemicon International). Slides were incubated with the following respective secondary antibodies: red fluorochrome-conjugated donkey anti-rabbit (1 : 1000; Alexa 568; Invitrogen) and green fluorochrome-conjugated donkey anti-mouse (1 : 1000; Alexa 488; Invitrogen). As a negative control, the primary antibodies were omitted. Nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI; Invitrogen) and slides were observed under a fluorescence microscope (Imager.Z1; Zeiss, Göttingen, Germany) and images captured (Carl Zeiss MicroImaging, Göttingen, Germany).

Quantitative immunostaining for eNOS

Immunodetection of eNOS was performed in penile tissues from late (8 weeks) diabetic and non-diabetic control groups using the streptavidin–biotin peroxidase method.²⁰ Briefly, CC sections were incubated overnight with the primary antibody, a cross-reactive rabbit anti-eNOS antibody (1 : 85; Abcam), followed by the secondary antibody (Santa Cruz Biotechnology) and the avidin–biotin complex reagent (Vector Laboratories, Burlingame, CA, USA). As a negative control, the primary antibody was omitted. Diaminobenzidine (DAB kit; Abcam) was used as the chromogen and sections were counterstained using hematoxylin. Immunostaining was visualized under an Olympus AH3-RFCA microscope and images were captured using an Olympus C-35AD-4 camera (Olympus Imaging Europa, Hamburg, Germany). Signal intensity was quantified as described previously²¹ using ImageJ color deconvolution (v.1.37a; National Institutes of Health, Bethesda, MD, USA). A DAB threshold value of 25 was selected for eNOS by measuring the lowest and highest mean optical density values in 10 random DAB-positive images. For each image, the sum of the intensity values in the regions of interest (ROI) was divided by the ROI total area, producing an average intensity unitless value for eNOS staining. All intensity values within the same group (non-diabetic and diabetic) were averaged to calculate an overall value, a relative measurement of eNOS protein levels.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad, San Diego, CA, USA). Data are presented as the mean \pm SE. The significance of differences between groups for changes in H_2O_2 production, the GSH/GSSG ratio, and quantitative immunostaining was calculated using Student's *t*-test. For Western blotting, significant differences between groups were determined using the one-way analysis of variance (ANOVA) followed by the Bonferroni test. Two-sided $P < 0.05$ was considered significant.

Results

Evaluation of systemic oxidative stress in non-diabetic and diabetic rats

To assess systemic redox status in early and late diabetes, urinary H_2O_2 levels and the plasma GSH/GSSG ratio were determined. There were significant increases in urinary H_2O_2 concentrations in samples from both early

(2 weeks) and late (8 weeks) diabetic rats compared with their respective age-matched controls ($P < 0.0001$ and $P = 0.042$, respectively; Fig. 1). In contrast, there were no significant changes in the GSH/GSSG ratio in early diabetic rats compared with the age-matched control (Fig. 2). However, the GSH/GSSG ratio decreased significantly in late diabetic rats compared with the age-matched control ($P = 0.029$; Fig. 2). The increase in H_2O_2 levels and decreased GSH/GSSG ratio with the progression of diabetes indicate an imbalance in the systemic redox status towards the promotion of oxidative damage.

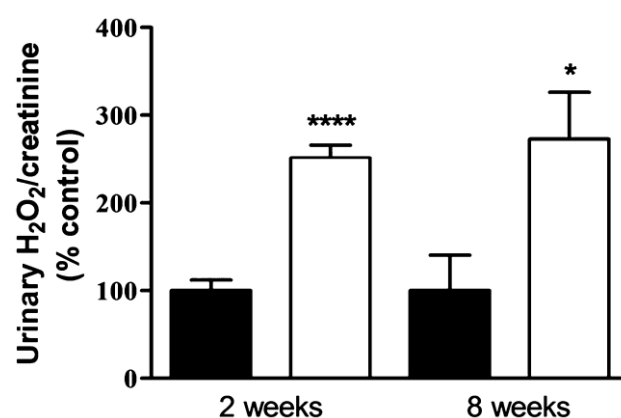


Figure 1 Systemic quantification of H_2O_2 levels in urine samples of 2 and 8-week diabetic rats and age-matched controls. Urinary H_2O_2 was significantly increased in both groups of diabetic rats when compared with respective age-matched controls. Data are the mean \pm SE. * $P < 0.05$, **** $P < 0.0001$ compared with control.

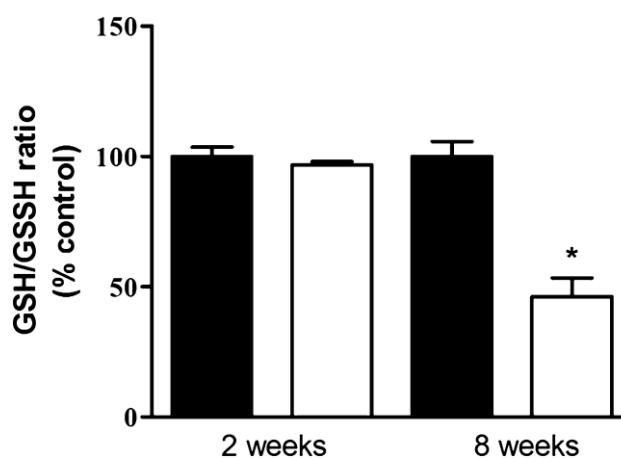


Figure 2 Chromatographic measurement of reduced/oxidized glutathione (GSH/GSSG) in the plasma of early and advanced diabetic animals. A significant decrease of plasmatic GSH/GSSG ratio was observed only in the plasma of 8-weeks diabetic rats. Data are the mean \pm SE. * $P < 0.05$ compared with control.

Local production of H₂O₂ by penile tissue

We next evaluated whether the presence of specific oxidative markers increased in diabetic cavernosal tissue with progression of the disease. There was a tendency for an increase in H₂O₂ release from penile tissue from early diabetic rats compared with age-matched controls, but the difference failed to reach statistical significance

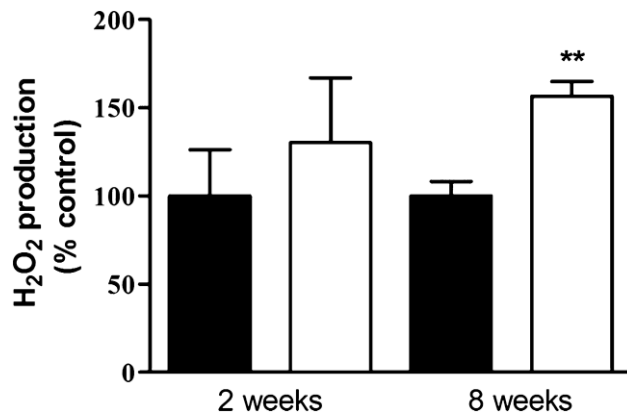


Figure 3 Penile measurement of H₂O₂ production in 2 and 8-week diabetic rats and age-matched controls. Data showed that at 2-weeks there was a non-significant increase in H₂O₂ release. A significant augmentation was verified only in penile H₂O₂ levels in the 8-weeks diabetic group, when compared with controls. Data are the mean ± SE. ***P* < 0.01 compared with control.

(Fig. 3). However, with progression of the disease, H₂O₂ production by erectile tissue increased significantly in the late diabetic compared with non-diabetic group (*P* = 0.0013; Fig. 3). Corroborating the results of systemic oxidative stress evaluation, these data suggest that, in penile tissue, H₂O₂-induced oxidative stress is mainly detected in the advanced stage of diabetes.

Assessment of penile 3-NT content in early and late staged diabetes

To obtain further insight into the noxious effects of oxidative stress in diabetic CC, we evaluated oxidative protein damage by detecting protein tyrosine nitration. Damaging changes to diabetic erectile tissue proteins were assessed by detection of 3-NT, a stable marker of protein oxidative damage. Expression of 3-NT was quantified by Western blotting and its cavernosal cellular localization was evaluated by double immunolabeling for 3-NT/α-SMA. As shown in Fig. 4a, three bands were identified for 3-NT formation, two corresponding to high molecular weight proteins (Bands A and B) and one corresponding to low molecular weight protein (Band C). There was no significant difference in cavernosal 3-NT between the early diabetic and age-matched control groups. However, there was a significant increase in protein nitration, particularly in proteins of Bands B and C, in late diabetic CC compared with the non-diabetic group (*P* < 0.01; Fig. 4b). Furthermore, signifi-

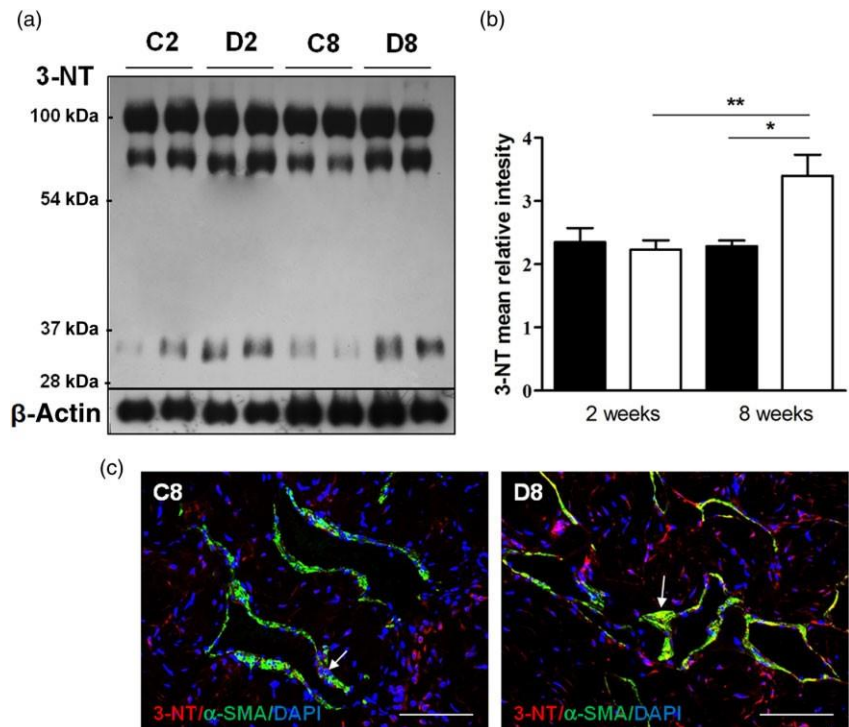


Figure 4 3-Nitrotyrosine (3-NT) protein quantification and expression pattern in control and diabetic cavernosal samples. (a, b) Western blotting results revealed a significant increase in penile protein oxidative damage in 8-week diabetic animals (D8) as compared to age-matched controls (C8) controls and to 2-weeks diabetic samples (D2). Densitometry and mean relative intensity quantification by comparing the intensity of 3-NT with the intensity of the β-actin protein. Data presented as mean relative intensity ± SE. ***P* < 0.01, **P* < 0.05. (c) 3-NT profile as assessed by double immunofluorescence for 3-NT and α-smooth muscle actin (α-SMA), revealed a perivascular smooth muscle and trabecular expression pattern for 3-NT. DAPI, 4',6'-diamidino-2-phenylindole (nuclear staining) (nuclear staining). Bars, 100 μm.

cant augmentation of protein nitration was detected with the progression of diabetes when comparing erectile tissue from late with early diabetic rats ($P < 0.05$; Fig. 4a,b). With regard to the pattern of 3-NT oxidative changes, there was abundant formation of tyrosine residues in diabetic cavernosal smooth muscle cells (SMC), as well as in the lining endothelium and trabecular fibroblasts (Fig. 4c). A similar pattern was found in non-diabetic CC (Fig. 4c), although with obviously lower 3-NT expression. These data corroborate the results obtained by Western blotting and reinforce the idea that increased penile protein oxidative modifications occur with the progression of diabetes. In addition to direct inactivation of NO when promoting 3-NT formation, a cavernosal reduction in NO may be related to changes in eNOS expression.

eNOS protein expression in diabetic cavernosal tissue

Changes in the activation of pathways involved in NO synthesis and/or bioavailability have been reported as consequence of hyperglycemia-induced oxidative stress.^{6,7} In addition, changes in eNOS may aggravate diabetic vascular-related events. Because oxidative stress has a more pronounced effect in late-stage diabetes, we quantified eNOS immunoreactivity staining in DAB-labeled sections 8 weeks after STZ injection using a color deconvolution method. In addition, we identified rat cavernosal components expressing eNOS using dual immunofluorescence for eNOS/ α -SMA. There was a significant decrease in eNOS signal intensity in late diabetic compared with age-matched control rats (39.25 ± 2.01 vs 50.34 ± 1.86 , respectively; $P = 0.0037$; Fig. 5a,b). With regard to the expression profile, eNOS in non-diabetic penile tissue was expressed in perivascular SMCs and particularly in the sinusoidal endothelium. A similar pattern was found in diabetic corporeal tissue (Fig. 5c). A decrease in eNOS expression indicates a reduction in diabetic cavernosal eNOS and impairment of vasorelaxation mechanisms, affecting erectile function.

Discussion

Diabetic men have an approximate threefold increased risk of the development of ED compared with non-diabetic individuals.^{2,3} It is known that hyperglycemia-induced increases in the production of AGEs, ROS, and RNS impair NO bioavailability and affect penile tissue, leading to changes in endothelium-dependent vasorelaxation mechanisms.⁵⁻⁷ In fact, mounting evidence indicates that oxidative stress plays a significant role in diabetes-associated ED.⁹⁻¹² However, basic research

studies in experimental models of diabetes have focused primarily on evaluating ROS production and the effects of antioxidant administration at one specific time point.^{7,9-12} Furthermore, and because oxidative stress involves multiple and complex mechanisms, there is a gap of information regarding the presence and function of oxidative stress changes in diabetic cavernosal tissue with the progression of the disease. It is the case of penile H_2O_2 production and the formation of 3-NT protein oxidative modifications. Therefore, we evaluated levels of cavernosal release of H_2O_2 , quantified 3-NT formation, and assessed changes in eNOS expression in early (2 weeks) and late (8 weeks) diabetes.

First, in order to characterize the overall redox status of diabetic rats 2 and 8 weeks after STZ injection, we quantified urinary production of H_2O_2 and evaluated cellular defense mechanisms by measuring the plasma GSH/GSSG ratio. The results reveal a significant increase in H_2O_2 levels in both the early and late diabetic groups. Because H_2O_2 , a powerful diffusible oxidizing molecule, is relatively stable in aqueous solution, direct measurement of H_2O_2 is relatively easy. Low levels of H_2O_2 have a physiological role in cell signaling. However, higher concentrations of H_2O_2 induce oxidative stress and contribute to cell damage. Primarily, H_2O_2 acts in an indirect manner in the presence of transition metal ions originating highly reactive hydroxyl radicals, which can damage DNA, lipids, and proteins.¹³ In fact, it has been reported that the measurement of urinary H_2O_2 may be a valuable biomarker for the detection of increased systemic oxidative stress associated with pathological conditions, including diabetes.²² In agreement with our results, previous studies have demonstrated augmentation of urinary H_2O_2 in STZ-diabetic 4 and 8 weeks after disease onset.²³ Accordingly, it is known that diabetes induces significant pathophysiological alterations with the progression of the disease, and therefore an increment of systemic oxidative stress is expected at later stages. Nonetheless, the observed increment in urinary H_2O_2 2 weeks after the onset of diabetes indicates that the production of this ROS may be an early oxidative event in diabetes.

Next, we investigated the plasma GSH/GSSG ratio in early and late diabetes. The GSH/GSSG ratio is the product of an antioxidant endogenous system consisting of enzymes with free radical-scavenging capacities, which oxidize GSH to GSSG and reduce the latter to GSH. Thus, GSH has a central role as an antioxidant and is the product of the action of glutathione reductase on GSSG.²⁴ Therefore, the plasma GSH/GSSG ratio can be an indicator of systemic redox status. The results of the present study revealed a significant reduction in the plasma GSH/GSSG ratio of late diabetic rats compared

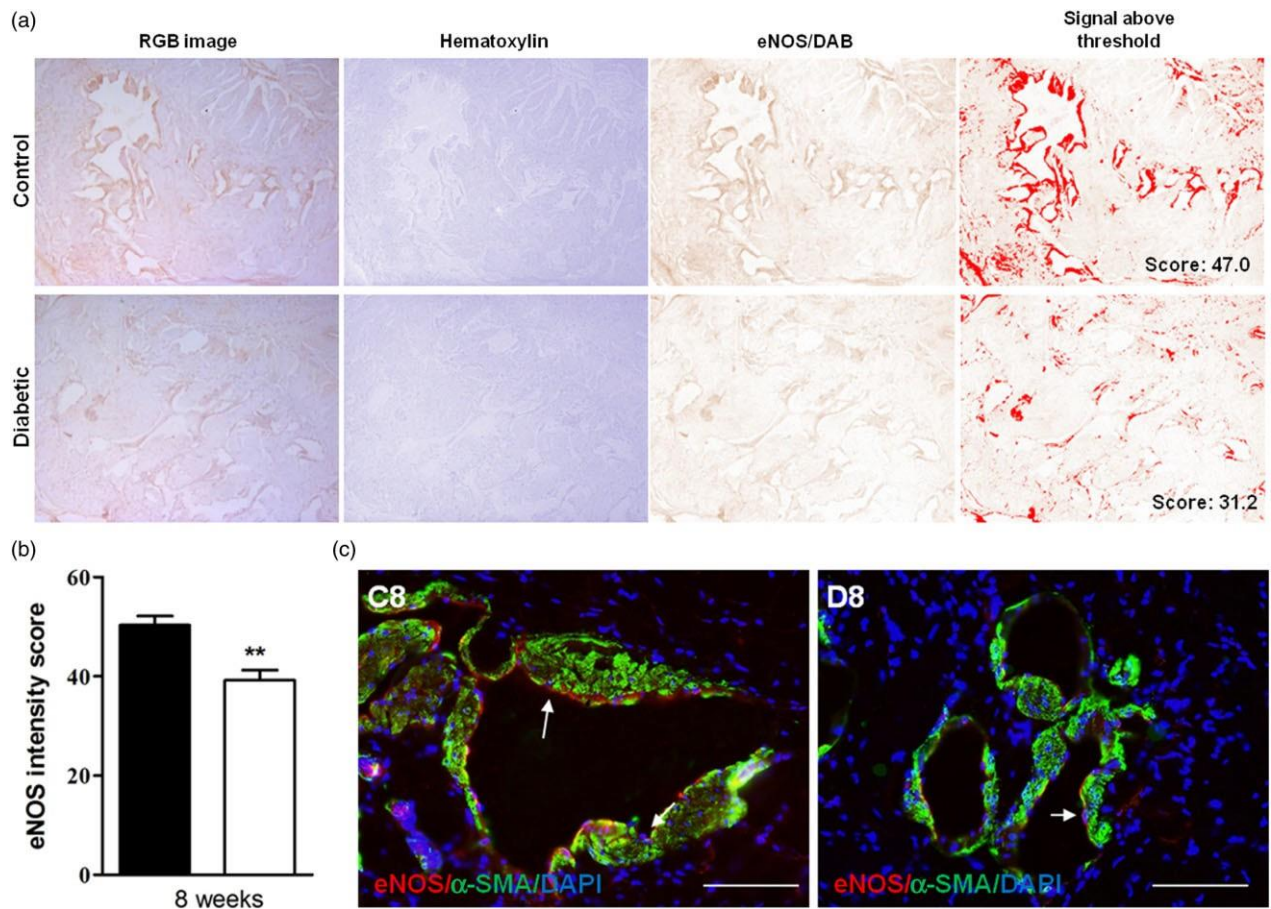


Figure 5 Quantitative immunohistochemistry of endothelial nitric oxide synthase (eNOS) in 2 and 8-week diabetic corporeal samples. (a,b) eNOS protein expression levels as quantified in eNOS-DAB stained slides by ImageJ color deconvolution, revealed a significant decrease in eNOS in diabetic corpus cavernosum (CC) compared with the age-matched control (39.25 ± 2.01 vs 50.34 ± 1.86 , respectively). Data presented as mean \pm SE. $**P < 0.01$ compared with control. RGB, red, green, blue RGB, xxx. Red Green Blue (RGB) (c) Colocalization of eNOS and α -smooth muscle actin (α -SMA) by dual immunolabeling demonstrated that eNOS protein was predominantly present in cavernosal endothelial cells and smooth muscle cells in samples from both non-diabetic and diabetic groups. DAPI, 4',6'-diamidino-2-phenylindole (nuclear staining); C8, control group at 8 weeks; D8, 8-week diabetics (nuclear staining); C8, control group at 8 weeks; D8, 8-week diabetics. Bars, 100 μ m.

with the non-diabetic age-matched control group, suggesting impairment of this system and an increment in systemic oxidative stress with the progression of diabetes. In fact, our data corroborate a previous study that also reported a decreased plasma GSH/GSSG ratio in a diabetic model.²⁵ According to our results, this antioxidant enzyme system appears to be altered as consequence of prolonged diabetes, thus exacerbating systemic oxidative stress.

After characterizing the systemic redox status in the early and late diabetic rats, we evaluated local cavernosal oxidative stress, namely the production of H_2O_2 and 3-NT formation, and eNOS expression. Despite data showing an increment of H_2O_2 levels in diabetic tissues,^{26,27} to date H_2O_2 production has not been evalu-

ated in diabetic CC with the progression of the disease. The results of the present study showed a tendency for an increase in cavernosal H_2O_2 release in early diabetes, which became statistically significant in late diabetes. Because H_2O_2 is a lipid-soluble molecule, it seems reasonable that systemically, in early diabetes, we already observe a significant increment in the urine and that in the penis, this increase becomes more prominent in the later stage of the disease. Tissue function is also affected by ROS-induced damage to proteins.¹⁴ In fact, 3-NT-modified proteins are susceptible to changes in structure, function, enzyme activity, and signaling pathway activation.^{14,15} Because of their role and because 3-NT protein modifications have not been evaluated thoroughly in cavernosal tissue with the progression of diabetes, we

quantified and characterized 3-NT expression in early and late diabetes. There was a significant increase in 3-NT-containing proteins of different molecular weights in late diabetic erectile tissue compared with the age-matched control and early diabetic groups. Corroborating our data, previous studies have reported an increment of penile 3-NT formation, as analyzed at single time points after the induction of diabetes, using immunohistochemical techniques.^{28–30} Dual immunofluorescence for α -SMA + 3-NT revealed a more prominent expression of 3-NT in SMCs, trabecular fibroblasts, and some labeling also in the lining sinusoids of late diabetic CC. This expression profile of 3-NT in diabetic erectile tissue is in agreement with previous reports,^{28,30} indicating that 3-NT formation may affect several cell compartments promoting tissue malfunction. Consistent with H₂O₂ data, more severe oxidative lesions in proteins were detected in late diabetes as consequence of sustained hyperglycemia and oxidative stress-induced molecular modifications.

In addition to the aforementioned changes, it is known that oxidative reactions involving ROS and RNS promote deregulations in NO bioavailability, either by directly rescuing it or by inducing changes in eNOS.^{6,31} It is well established that impairment in NO physiology alters penile vascular tone and relaxation of corporeal smooth muscle, hampering penile vasorelaxation and erection.^{6,7} Because more prominent changes were observed at the later time point of diabetes, we evaluated eNOS protein expression in diabetic cavernosal tissue by quantitative immunohistochemistry in rats 8 weeks after the induction of diabetes. There was a significant reduction in eNOS protein expression in penile tissue of late diabetic rats. Although this quantification method has not been used to quantify eNOS in CC previously, it was found to be a reliable tool because our data corroborate previous publications in which decreased eNOS expression was verified by different techniques.^{18,32} Further, we evaluated the eNOS expression pattern and detected specific signals in the cavernosal endothelium and in the SMC component, in agreement with recent studies.^{18,32}

Overall, we observed an increase in systemic and local oxidative status in penile tissue with the progression of diabetes. As the disease progressed, we detected an increment in cavernosal H₂O₂ production, increased oxidative injury to proteins, and changes in eNOS protein expression. These may hamper erectile tissue cellular and/or molecular function and vasorelaxation events, contributing to ED progression.

The results of the present study demonstrate that the noxious effects of oxidative stress are more prominent in advanced stages of diabetes. We observed an imbalance in systemic oxidative stress mechanisms, with an increase

in H₂O₂ production and impairment in the endogenous antioxidant GSH/GSSG system. Locally, in diabetic penile tissue, we detected a marked increase in oxidative markers, namely H₂O₂, and augmentation of oxidative stress-induced protein modifications, which may be responsible for promoting structural and/or functional changes in cellular and/or molecular mechanisms essential for the normal erectile process. A reduction in eNOS expression was also observed with the progression of diabetes, indicating a deregulation in vasorelaxation mechanisms.

Overall, increased cavernosal oxidative stress, augmented protein oxidative damage, and decreased eNOS expression highlight the role of deleterious events induced by diabetes that contribute to ED and strengthen the relevance of preventative antioxidant therapy.

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Disclosure

None declared.

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